Free Radicals and Antioxidants, 2016; 6(2): 139-144
A multifaceted peer reviewed journal in the field of Free Radicals and Antioxidants
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Original Article

Comparative Study of Phytochemicals, Antioxidative Potential & Activity of Enzymatic Antioxidants of *Eclipta alba* and *Plumbago zeylanica* by *in vitro* Assays

Abhilasha Saxena[1,2], Sanghpriya Gautam[3], Kamal Ram Arya[3], Rama Kant Singh[1,2*]

[1]Academy of Scientific and Innovative Research (AcSIR), Anusandhan Bhawan, New Delhi, INDIA.
[2]Division of Toxicology, CSIR-Central Drug Research Institute, Lucknow, Uttar Pradesh, INDIA.
[3]Division of Botany, CSIR-Central Drug Research Institute, Lucknow, Uttar Pradesh, INDIA.

ABSTRACT

Background: The Plumbago *zeylanica* (family: Plumbaginaceae) is a widely accepted ethnomedicine around the world including India, Pakistan, Bangladesh, Sri Lanka, and Australia. Early *in vitro* researches of *Plumbago zeylanica* were focused on antioxidant activity in root only a few were concerned with stem. Similarly in *Eclipta alba* antioxidant activity of whole plant is explored. **Objective:** The present study highlights the comparative antioxidant potential and enzymatic contents of both plants along with recent updates on phytochemical contents. **Materials and Methods:** The antioxidant potential of the plants was established by DPPH free radical scavenging assay, Nitric oxide (NO) free radical scavenging activity, Hydrogen peroxide (H_2O_2) scavenging activity, Catalase activity along with determination of Total Phenolic Contents in the plant extracts. **Results:** The results revealed that *Eclipta alba* has notable activity than *Plumbago zeylanica* in quenching of DPPH while *Plumbago zeylanica* shows comparatively better activity in nitric oxide scavenging, super oxide scavenging and Catalase activity. The *Eclipta* at 10 µg/ml showed maximum scavenging of DPPH (16.60 %), nitric oxide (66.21%), super oxide (4.76%) and Catalase activity 31.9 at 1 mg/g fresh weight against the *Plumbago zeylanica* which showed maximum scavenging of DPPH 13.24%, Polyherbal extract 11.78%, nitric-oxide (69.45%), super oxide (5.12%) and catalase activity 23.7 at 1 mg/g fresh weight respectively at the same concentration. The phytochemical screening of the extract revealed the presence of considerable amount of alkaloids, saponins, tannins, phenolic compounds and glycosides which might be responsible for the antioxidative potential of the selected plants. **Conclusion:** The results of this study strongly indicate that the *Plumbago zeylanica* has comparatively more potent antioxidant potential than *Eclipta alba*. Moreover, the study reveals that polyherbal extract for the very first time for this combination have shown a strong antioxidant activity which is directly correlated with suppression of free radical induced diseases i.e. cancer. These findings encourage studying these plants and their polyherbal extracts further as a potential agents against cancer.

Key words: Antioxidant activity, *Eclipta alba*, Enzymatic contents, Phytochemical screening, *Plumbago zeylanica*.

Correspondence :

Dr. Rama Kant Singh,
Senior Principal Scientist & Incharge,
Department of Toxicology,
CSIR-Central Drug Research Institute,
BS-10/1,Sector-10, Jankipuram Extension,
Sitapur Road, P.O.Box 173, Lucknow-226031,
Uttar Pradesh, INDIA.
Phone no: 9984941595

E-mail: rktox@yahoo.com; rk_singh@cdri.res.in
DOI: 10.5530/fra.2016.2.2

INTRODUCTION

Eclipta alba (L.) Hassk. belonging to family Asteraceae is commonly known as false daisy or Bhingaraj in the Indian system of medicine. It is a creeping herb which grows in moisture is usually found on roadsides and wastelands in all over India.[1] Various studies have been carried out to indicate that flavonoids and other polyphenolic compounds have a major role to play for antioxidant activity.[2] The whole plant is widely utilized for its stimulant properties. The flowers possess diverse properties like analgesic, antispasmodic, fungicidal, digestive, bactericidal and vulnerary. The plant also posseses some important pharmacological activities such as antimicrobial, hepatoprotective, antioxidant, anti-antiviral, inflammatory, immunomodulatory and analgesic activity.[3] As free radicles play a crucial role in the pathogenesis of cancer, this oxidative damage along with progression of the disease could be arrested by utilizing the strong antioxidant defense mechanism possessed by these excellent natural source of antioxidants which helps to reduce the risk of free radical induced diseases[4-6] without having side effects which has shifted the focus from traditional medicines to alternative forms of therapy based on natural sources.[7] The plant *Plumbago zeylanica* belongs to family Plumbaginaceae of genus Plumbago which consists of 10-20 flowering plants species. The *P. zeylanica* (White Leadwort) plant is found in warm temperate-tropical regions of the world and grows wild in India (especially in Bengal, Uttar Pradesh, and South Indian states) and Sri Lanka.[8] It is supposed to be originated in South-East Asia.[9,10] It has been used by rural and tribal people for hundreds of years as traditional medicine.[11] Roots of plant possess various therapeutic properties like, hepatoprotective, neuroprotective, anti-atherogenic, anti-anthrogenic, cardiotonic, hepatoprotective and neuroprotective properties.[12] The anticancer, antibacterial, antifungal and antitumor properties of the plant are already reported.[13] The leaves and roots of *P. zeylanica* contains an alkaloid called plumbagin (2-methoxy-5 hydroxy-1, 4-napthoquinone).[1] The plant has great potential for various diseases and disorders along with great antioxidant activity. In the present investigation aimed to evaluate the comparative antioxidant potential of *Eclipta alba* and *Plumbago zeylanica* by *in vitro* assays as a possible mechanism for antileukemic effect.

MATERIALS & METHODS

Collection and Identification

The plants so obtained from their relevant sources were authenticated by Dr. K.R. Arya, Principal Scientist and In-charge, Botany Division, CSIR-Central Drug Research Institute Lucknow (U.P.), India and are kept at medicinal plant repository of the institute with Identified Plant Sample Number KRABOT 24501 for *Eclipta alba*, KRABOT 24502 for *Plumbago zeylanica*.

Chemicals

Chemicals like DPPH (1, 1-Diphenyl-2-picrylhydrazyl) and Griess reagent were procured from Sigma-Aldrich Chemicals. Folin Ciocalteau reagent and Sodium carbonate were procured from Himedia Lab. Pvt. Ltd. Sodium nitroprusside, Fehling's solution A & Fehling's solution B, Benedict's reagent, $FeCl_3$, Mayer's Reagent, Dragendorff's Reagent, Biuret reagent, H_2SO_4, $KMNO_4$, KNO_3 were procured from Loba Chemie Pvt. ltd, Mumbai, India. Chloroform, Gallic acid, and Hydrogen peroxide (H_2O_2), NaH_2PO_4 and Na_2HPO_4 were purchased from Merck Specialities Pvt. Ltd and Methanol was procured from Molychem.

Preparation of Crude Extract of Plants

The plants materials after collection and authentication were shade dried and coarsely powdered with the help of mechanical grinder. Crude ethanolic extracts of all the plant materials were made following a standard protocol explained here under -

The whole plant material was dipped in ethanol for 24 h with intermittent shaking 2-3 times then it was filtered using 125 mm Whatman qualitative filter paper under sterile conditions and the filtrate was collected in a separate conical flask. After percolation the filtrate was concentrated in a round bottom flask using rotavapour at 40°C. This process is repeated 4 times unless the filtrate becomes colourless. Finally the concentrates in a round bottom flask were stored in fridge at 4°C.

Preliminary Qualitative Phytochemical investigation

Preliminary phytochemical investigation was performed on the ethanolic extract of *Eclipta alba* and *Plumbago zeylanica* using qualitative tests to identify the phytoconstituents in the extract by the method of.[14]

Determination of Total Phenolic Content

The total phenolic content of the plant extracts was analyzed spectrometrically by the method of.[15] 1 ml of each extract was taken. In it 1 ml of Folin-Ciocalteu reagent was added and incubated for 5 min. 10 mL of 7% (w/v) sodium carbonate solution was added in it followed by the addition of 13 ml distilled water. The mixture was kept in dark for 90 min at 23°C. Finally, absorbance was measured at 750 nm. For each concentration the samples were prepared in triplicate. Then the content of phenolics in extracts was expressed in terms of Gallic acid equivalent (mg of GA/g of extract).

DPPH radical scavenging assay

The DPPH radical scavenging assay was conducted following the method of Brand Williams *et.al.*[16] In brief, 1 mL of DPPH solution (0.1 mM in methanol) was blended with plant extract solution at varying concentrations 0.5, 1, 5 and 10 μg/ml in triplicate. Mixer of 1 ml methanol and 1 ml of DPPH solution was used as control. As the next stage, the reaction mixture was shaken and incubated in the dark at room temperature for 30 min, and the absorbance was read at 517 nm against the blank using UV-VIS spectrophotometer. The inhibition of the DPPH radical by the sample was calculated based on the formula below.

$$Inhibition\ \% = \frac{Ac\text{-}As \times 100}{Ac}$$

Ac=Absorbance of the control

As=Absorbance of the sample

Nitric oxide (NO) free radical scavenging activity

NO scavenging activity was determined according to the method of *Garrat,* 1964. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates NO. 2 ml of 10 mM sodium nitroprusside in 0.5 ml of PBS was mixed with 0.5 ml of extract at various concentrations in triplicate and the mixture was incubated at 23°C for 150 min. 0.5 ml was taken out from the incubated solution and added into 1 ml of Griess reagent and incubated at RT (23°C) for 5 min. The absorbance at 540 nm was measured with a spectrophotometer.[17]

The NO radical scavenging activity was calculated according to the following equation:-

$$\%\ NO\ inhibitory\ activity = \frac{Ao - As \times 100}{Ao}$$

Where Ao=Absorbance of the control (blank, without extract); As=Absorbance in the presence of the extract.

Hydrogen peroxide (H_2O_2) scavenging activity

The capability of scavenging hydrogen peroxide by the extract was determined based on the method of Ruch *et al.* 1989.[18] 3.4 ml of phosphate buffer pH 7.4 was added to 43 mM hydrogen peroxide solution to which 1 ml of each of plant extract in triplicate was added and tubes were vortexed and the absorbance was measured at 230 nm after 10 min, against a blank. The abilities to scavenge the hydrogen peroxide were calculated based on the following equation:

$$\%\ Hydrogen\ peroxide\ inhibition = \frac{Ao\text{-}As \times 100}{Ao}$$

Where Ao=absorbance of the blank without extract (control); As=absorbance in presence of extract.

Catalase activity

Catalase activity was assayed following to the method prescribed by Euller & Josephson[19] with some recent modifications for the estimation of catalase activity by Bisht.[20] 5% extract of plant tissue was prepared. The catalase activity was assayed by taking two test tubes for each sample, one for catalase activity and the other for its blank. In each test tube 2 ml citrate phosphate buffer (pH 7.0) was added then 2 ml 0.5% H_2O_2, 2 ml D.W. 2 ml tissue extract was added in a series and incubated for 10 min. Thereafter, the reaction was stopped by adding 2 ml 4N H_2SO_4 and thus was done in the case of sample showing catalase activity and in the case of blank, H_2SO_4 was added before addition of H_2O_2, finally titrated against 0.01N $KMnO_4$.

Catalase activity was calculated as follows:

$$Blank - Sample \times 40$$

Activity was measured in terms of ml H_2O_2 hydrolyzed/g fresh weight

Statistical analysis

All the experiments were conducted in triplicates and data given in tables are average of the three replicates and statistically analyzed for the calculation of standard error (SE), finally tested with using Students't' test for significance. $P<0.05$ was considered as significant.

RESULTS

Preliminary phytochemical investigation of the ethanolic extract revealed the presence of alkaloids, saponins, tannins, phenolic compounds and glycosides as the phytoconstituents present in the plant (Table 1; Figure 1). The total Phenolic Content was estimated by using Folin-Ciocalteu's reagent. Total Phenolic Content of the different extracts of *Eclipta* & *Plumbago* was solvent dependant and expressed as mg of Gallic acid equivalent (GAE). Table 2 summarizes that the total phenolic compound in extract varied widely ranging from (2.901 ± 1.3; 20.32 ± 1.9 mg/g) in *E. alba* and (3.25 ± 1.8; 22.66 ± 2.3 mg/g) in *P. zeylanica* expressed as Gallic acid equivalent (GAE) but ethanol and water extract exhibited the highest total phenolic content respectively.

Table1: Tabular presentation of the Phytoconstituents present in *Eclipta alba* **&** *Plumbago zeylanica*

1.Test for Carbohydrates	*Plumbago zeylanica*	*Eclipta alba*
a. Fehling's test	Absent	Absent
b. Benedict's test	Absent	Absent
2. Test for Alkaloids		
a. Mayer's test	Present	Present
b. Dragendroff's test	Present	Present
3. Test for Saponins	Present	Present
4. Test for Tannins	Present	Present
5. Test for Phenolic compounds	Present	Present
6. Test for flavonoids	Absent	Absent
7. Test for Amino acids		
a. Biuret test	Absent	Absent
b. Xanthoproteic test	Present	Absent
8. Test for Glycoside	Present	Present
9. Test for Steroids/ Terpenoids	Absent	Absent

Table 2: Total Phenolic content in methanol and Ethanol extract of *Eclipta alba* and *Plumbago zeylanica*

Plant extracts	Name of the Plant	Total Phenolic Content (mg Gallic acid Equivalent/g)
Ethanolic	*Eclipta*	2.90 ± 1.3
	Plumbago	3.25 ± 1.8
Methanol	*Eclipta*	16.29 ± 2.8
	Plumbago	19.30 ± 2.9
Ethanol + Water	*Eclipta*	20.32 ± 1.9
	Plumbago	22.66 ± 2.3

± S. E. Value (n=3);*-value significant at p<0.05 level.

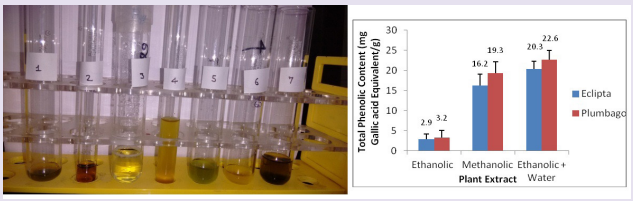

Figure 1: Phytochemical Screening tests of *Eclipta alba*
1=Carobhydrate, 2=Alkaloid(Dragendroff's test), 3=Saponin, 4=Tannin, 5=Phenolic compound, 6= Flavonoid, 7=Amino acid (Xanthoproteic test).

DPPH radical scavenging assay

In the results of the present study, a % increase in scavenging effect was observed with increasing concentrations of the plant extract taken as 0.5, 1, 5, 10 µg/ml respectively. *Eclipta alba* showed higher scavenging effect of 16.60% at higher conc. of 10 µg/ml with an IC_{50} of 39.35µg/ml as compared to *Plumbago zeylanica* showing an average 13.24 % scavenging effect at same conc. which is slightly less than *Eclipta alba* with an IC_{50} of 55.41µg/ml. While a wonderful effect of DPPH was observed on the combination of *Eclipta alba* and *Plumbago zeylanica* which again showed an increase in scavenging effect in the same manner as with concentration of the plant extracts taken with a % scavenging effect of 11.78% with an IC_{50} of 61.03 µg/ml which is first time studied by our group. While the % of scavenging of the ascorbic acid was 33.45 % at higher conc. with an IC_{50} of 15.23 µg/ml. The results obtained were comparative to standards used (methanol) in blank which served as control (Figure 2).

Nitric oxide (NO) free radical scavenging activity

Effect of different concentrations of ethanolic extract on nitric oxide scavenging capacity was determined and results are presented in Figure 2. The extract of *E. alba*, exhibited scavanging potential in an increasing order with respect to the concentration of the plant extract taken. A % increase in scavenging effect was observed with increasing concentrations of the plant extract taken as 0.5,1,5, 10 µg/ml respectively. *Eclipta alba* showed an IC_{50} of 6.189 µg/ml as compared to *Plumbago zeylanica* showing an IC_{50} of 5.836 µg/ml as compared to the control ascorbic acid with an IC_{50} of 3.50 µg/ml (Figure 3).

Hydrogen peroxide (H_2O_2) scavenging activity

The plant extract was capable of scavenging H_2O_2 in a dose-dependent manner. *Eclipta* exhibited 4.76% scavenging activity on H_2O_2. On the other hand, at the same concentration *Plumbago* showed 5.123 % scavenging effect which are correlated to the standard with highest scaveng-

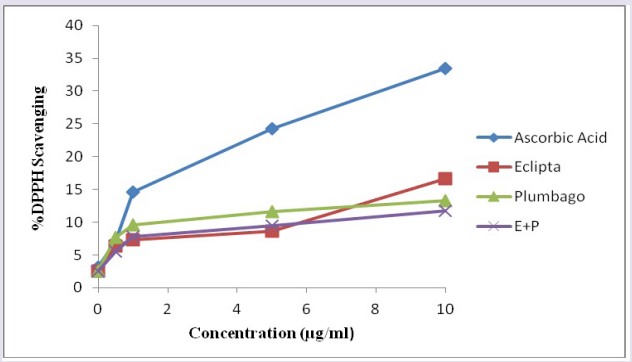

Figure 2: DPPH Free Radicle Scavenging Activity in *Eclipta alba*, *Plumbago zeylanica* & E+P. ± S. E. Value (n=3);*-value significant at p<0.05 level.

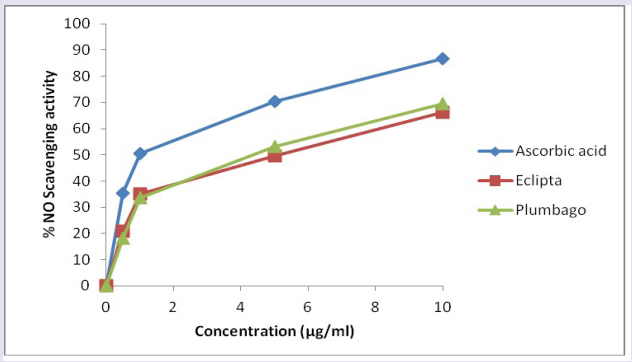

Figure 3: Nitric Oxide Scavenging Activity in *Eclipta alba* & *Plumbago zeylanica*. ± S. E. Value (n=3);*-value significant at p<0.05 level.

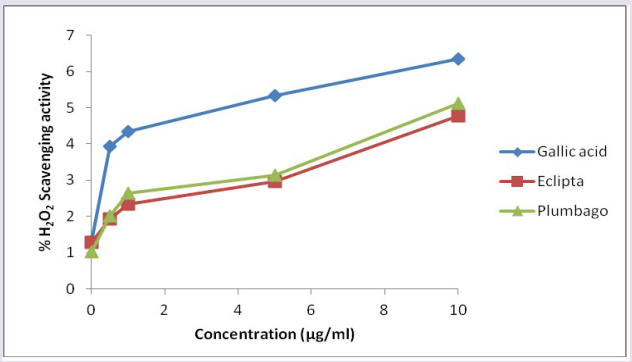

Figure 4: Hydrogen Peroxide Scavenging Activity in *Eclipta alba* & *Plumbago zeylanica*. ± S. E. Value (n=3);*-value significant at p<0.05 level.

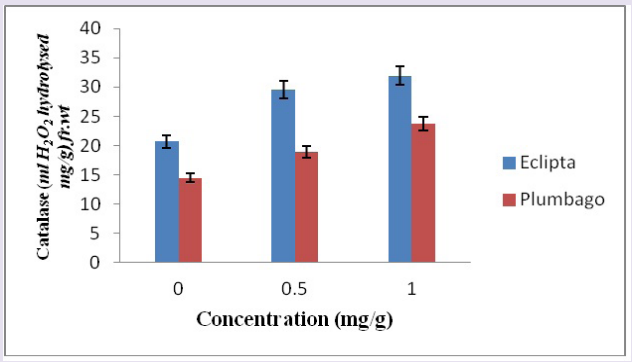

Figure 5: Catalase activity in *Eclipta alba* & *Plumbago zeylanica*. ± S. E. Value (n=3);*-value significant at p<0.05 level

ing effect of 6.35% at 10µg/ml with an IC_{50} value of 128.21 µg/ml. These results indicated that *Plumbago* posses effective H_2O_2 scavenging activity (Figure 4).

Catalase activity

The results reveal an increase in the catalase activity of 29.5 and 39.1 at 0.5 and 1 mg/g fresh wt respectively in *Eclipta alba* and 18.9 and 23.7 at the same concentrations in *Plumbago zeylanica* as compared to control (Figure 5).

DISCUSSION

Antioxidant therapy is an exciting new concept in the prevention and treatment of countless human diseases such as inflammation, cardiovascular disease, cancer and aging related disorders which originate from free radical derived oxidative stress. In this respect, natural compounds are again of growing interest and became intensively investigated within the last years. Hence, with this ongoing discussion the present work is envisaged to explore the comparative antioxidative potential of the selected plants along with their poly herbal extract for the very first time for this combination which has shown a strong antioxidant activity and is now capable of mentioning its strong capability to cure these diseases without having any side effects. Presence of the strong antioxidant activity in the poly herbal extract at such a lower concentration is in itself a novel finding as it is directly correlated with suppression of free radical induced diseases i.e. cancer. This will also give new opening by providing a new lead for further research in this direction for exploring more natural products having antioxidant action. Moreover the present study well elucidated antioxidant potential of stem of *plumbago zeylanica* against whole plant extract of *Eclipta alba* which is different from previous studies as much of work is emphasized on root of *Plumbago.*[21-24] The powerful scavenging of free radicles by these plants could be due to synergistic effect and the key contribution of many phyto-constituents together in the extract for achieving the desired therapeutic effects.[25] The present study leaves a tremendous scope for finding out the underlying mechanism responsible for this potential.

It is reported that phenols play a key role for the variation in the antioxidant activity of the plant.[26] They execute their antioxidant activity by two ways i.e lipid free radicals are inactivated or hydroperoxides are prevented from decomposition into free radicles.[27,28] It is well known that the antioxidant effect of plant products is mainly due to radicals scavenging activity of phenolic compounds such as tannins, flavonoids, polyphenols, and phenolic terpenes.[29] In the present study, analysis of Total Phenolic content showed that Methanol and ethanol extract of whole plant of *Eclipta* and stem of *Plumbago* can be potent source of natural antioxidant. Whereas ethanolic and water extract exhibited highest total Phenolic content in both plants. The methanol extract showed highest total Phenolic content in *Ficus microcarpa* L.[30] These results indicated that the extracts have a notice-able effect on scavenging the free radicals. Among solvents used in this study, Ethanol has showed the best effec-

tiveness in extract showing Phenolic components. Ethanol is preferred for the extraction of antioxidant compounds mainly because its lowers toxicity.[31]

DPPH solution executes a deep purple colour when prepared fresh having maximum absorption at 517 nm. The colour developed later on fades as free radicles of DPPH are quenched by the molecules of antioxidants and converting them into a colourless-/bleached product which leads to decrease in absorbance at 517 nm band.[32] Results were found that the ethanolic extract of *Eclipta alba* possessed significant antioxidant activity when compared to the ethanolic extract of *Plumbago zeylanica*. The IC_{50} value of *Eclipta* was slighltly less as compared to the IC_{50} values of *Plumbago*. In another studies for the same parameter *Eclipta alba* (82.51%) showed potent activity at the concentration of 100 µg/ml than compared to standard ascorbic acid.[33]

Another study confirmed that *Eclipta Prostrata* (L.) extract has good antioxidant property was assessed by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical.[34] Other findings reported IC_{50} values of 21.35 µg/ml and 40.87 µg/ml were obtained from ethanolic extract and ascorbic acid respectively. Ethanolic extracts of *E. alba* exhibited better antioxidant potential in comparison to ascorbic acid as evidenced by lower IC_{50} values respectively in DPPH assay.[35] Similarly, DPPH assay was employed for *Plumbago* in a study which revealed the antioxidant activity by DPPH is 96 µg/ml and by NBT is 4.6 µg/ml which is greater than that of standard (Quercetin) 45 µg/ml by DPPH and 10µg/ml by NBT assay.[21]

The results of Nitric oxide radical scavenging assay showed an IC_{50} of 6.189 µg/ml *in Eclipta alba* as compared to *Plumbago zeylanica* showing an IC_{50} of 5.836 µg/ml as compared to the control ascorbic acid with an IC_{50} of 3.50 µg/ml. Hence it can be said that *Eclipta alba* not only quenches free radicals but also efficiently inhibits reactive nitr--ogen species. In another studies for the same parameter, *E. alba* exhibited scavenging potential with IC_{50} values of 17.55 µg/ml. These values were significantly lower than ascorbic acid (46.67 µg/ml) used as standard in the assay indicating higher antioxidant activity of extracts of *E. alba*.[35] Other studies revealed the average IC_{50} of the methanolic extract was found to be 2.871µg/ml and that of the hydrolyzed extract was found to be 5.347 µg/ml.[25]

The H_2O_2 scavenging capacity of an extract may be credited to the structural features of their active components of the extract which decides their electron donating abilities.[36] The plant extract of our study were capable of scavenging H_2O_2 in a dose-dependent manner. *Eclipta* exhibited 4.76% scavenging activity on H_2O_2. On the other hand, at the same concentration *Plumbago* showed 5.123 % scavenging effects which are correlated to the standard with highest scavenging effect of 6.35% at 10 µg/ml with an IC_{50} value of 128.21 µg/ml. In previous studies it was reported that methanolic extract of *Eclipta* leaf expressed the better scavenging activity (70%) for H_2O_2.[37] The IC_{50} value of the methanolic extract of *Eclipta alba* and standard ascorbic acid for hydrogen peroxide scavenging was found to be > 1000 µg/ml and 64.67 ± 1.15 µg/ml respectively.[38]

Catalase activity, are presented in Figure 3. High concentrations of catalase activity are observed in (23.7 and 31.9 mg/g tissue) in *Plumbago* and *Eclipta* leaves. Hydrogen peroxide is generated by the dismutation of superoxide radical by the enzyme superoxide dismutase. The H_2O_2 causes cell membrane damage leading to release of arachidonic acid is a long acting cell damaging molecule by Oberley and Oberley.[39]

CONCLUSION

The present study compared the Phytochemicals, Antioxidative potential and Activity of Enzymatic antioxidants of *Eclipta alba* and *Plumbago zeylanica* and strongly indicated that the *Plumbago zeylanica* has comparatively more potent antioxidant potential than *Eclipta alba. Moreover,* the study reveals that polyherbal extract for the very first time for this combination have shown a strong antioxidant activity which is directly correlated with suppression of free radical induced diseases i.e. cancer. These findings encourage studying these plants and their polyherbal extracts further as a potential agents against cancer.

ACKNOWLEDGMENTS

The authors are thankful for the support from Director C.D.R.I, for providing necessary funds for the study and thankful to Dr. M. N. Srivastava, Senior Principal Scientist, Botany Division, CSIR-CDRI for their kind support to perform the study.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

ABBREVIATION USED

DPPH: 2,2-Diphenyl-1-picrylhydrazyl; **NO:** Nitric oxide; **H_2O_2:** Hydrogen Peroxide.

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SUMMARY

- The results of this study strongly indicate that the *Plumbago zeylanica* has comparatively more potent antioxidant potential than *Eclipta alba*.
- The study reveals that polyherbal extract for the very first time for this combination have shown a strong antioxidant activity which is directly correlated with suppression of free radical induced diseases i.e. cancer.
- The phytochemical screening of the extract revealed the presence of considerable amount of alkaloids, saponins, tannins, phenolic compounds and glycosides which might be responsible for the antioxidative potential of the selected plants.
- These findings encourage studying these plants and their polyherbal extracts further as a potential agents against cancer.

ABOUT AUTHORS

Dr. Rama Kant Singh: Is Senior Principal Scientist & Incharge of Toxicology Division, C.D.R.I., Lucknow. He has 34 years of research experience of toxicology of drugs, vaccines and herbal products. He is also working as a guest faculty of Environmental Science in University of Lucknow for the past 25 years.

Abhilasha Saxena: Is Senior Research Fellow in C.D.R.I, Lucknow pursuing her Ph.D under the supervision of Dr. R.K. Singh. She is working on Evaluation of in vitro antileukemic and antioxidant activities of plant extracts.